Blastocele fluid from in vitro– and in vivo–produced equine embryos contains nuclear DNA

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Abstract: Normal mammalian early embryonic development involves apoptosis of blastomeres as a remodeling process during differentiation, starting at the blastocyst stage. Genomic DNA has been recently detected in the blastocele fluid of human embryos and has been amplified by real-time polymerase chain reaction (PCR) to diagnose the sex of in vitro-produced human embryos. This new approach varies from conventional preimplantation genetic diagnosis in that no cells are extracted from the embryo and only the blastocele fluid is aspirated and used as a DNA sample for diagnosis. In the present work, we investigated whether the blastocele fluid of equine preimplantation embryos contains nuclear DNA and whether this DNA could be used to diagnose the sex of the embryos by conventional PCR, using specific primers that target the TSPY and AMEL equine genes. The sex of 11 of 13 in vivo-produced embryos and of four of five in vitro-produced embryos was successfully diagnosed. The PCR amplification product was analyzed using genetic sequencing reporting that the DNA present in blastocele fluid was genomic. Additionally, after polyacrylamide gel electrophoresis and silver staining, the blastocele fluid from three different embryos produced a ladder pattern characteristic of DNA fragmented during apoptosis. Therefore, the results presented in this work report that blastocele fluid from in vivo- and in vitro-produced equine embryos contains nuclear DNA which is probably originated by apoptosis of embryonic cells, and this DNA could be used to diagnose the sex of preimlpantation embryos by conventional PCR

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Blactocoele fluid from In Vitro and In Vivo produced equine embryos contain nuclear DNA

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Blactocoele fluid from In Vitro and In Vivo produced equine embryos contain nuclear DNA

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Abstract

Normal mammalian early embryonic development involves apoptosis of blastomeres as a remodeling process during differentiation, starting at the blastocyst stage. Genomic DNA has been recently detected in the blastocoele fluid of human embryos and has been amplified by real-time PCR to diagnose the sex of in vitro produced human embryos. This new approach varies from conventional Preimplantation Genetic Diagnosis (PGD) in that no cells are extracted from the embryo and only the blastocoele fluid is aspirated and used as a DNA sample for diagnosis. In the present work, we investigated whether the blastocoele fluid of equine preimplantation embryos contain nuclear DNA and if this DNA could be used to diagnose the sex of the embryos by conventional PCR, using specific primers that target the *TSPY* and *AMEL* equine genes. The sex of 11 out of 13 in vivo produced embryos and of 4 out of 5 in vitro produced embryos was successfully diagnosed. The PCR amplification product was analyzed using genetic sequencing demonstrating that the DNA present in blastocoele fluid was genomic. Additionally, after polyacrylamide gel electrophoresis and silver staining, the blastocoele fluid from three different embryos produced a ladder pattern characteristic of DNA fragmented during apoptosis. Therefore, the results presented in this work demonstrate that blastocoele fluid from in vivo and in vitro produced equine embryos contain nuclear DNA which is probably originated by apoptosis of embryonic cells and this DNA could be used to diagnose the sex of preimlpantation embryos by conventional PCR.

Keywords

Preimplantation Genetic Diagnosis, Equine, Preimplantation embryo, Sex determination, Apoptosis.

1. Introduction

Apoptosis, a form of cell death that affects isolated cells, is characterized by nucleus and DNA fragmentation, cytoplasm condensation, membrane changes and cell death without lysis or damage to neighboring cells. It is a normal physiological phenomenon that occurs in multicellular organisms and is genetically determined.

During early development of mammalian embryos, the blastomeres differentiate at the blastocyst stage into two different cell lineages: the trophoblast, which will give rise to extraembryonic tissue, and the inner cell mass, which forms the fetus. In a normal developing embryo, these two cell lineages undergo apoptosis [1], a process that occurs as a mechanism to eliminate unwanted or genetically defective cells.

Recently, Palini et al. [2] reported for the first time the presence of genomic DNA in the blastocoele fluid of in vitro produced human embryos. These authors demonstrated that this DNA was amplifiable by real-time PCR and hypothesized that this DNA could be released into the blastocoele cavity from cells undergoing apoptosis. In addition, they were able to determine the sex of 26 out of 29 human embryos by amplifying the DNA present in the blastocoele fluid from these embryos.

Preimplantation Genetic Diagnosis (PGD) allows the identification of specific genetic traits of early embryos. Preimplantation Genetic Diagnosis was first used in humans more than twenty years ago [3] and has also been used in bovine [4], caprine [5] and equine [6] preimplantation embryos. Conventional PGD involves obtaining blastomeres by biopsy from early embryos, which has to be performed carefully in order to avoid impairing the viability of the embryo. Blastocoele fluid aspiration is a simpler technique to obtain genomic DNA from embryos since only fluid is collected from the blastocysts and therefore, if it can be used to determine the sex of preimplantation embryos efficiently, gender by PGD could be determined faster. This is important if PGD is performed to avoid transferring embryos of the undesired sex, since the determination needs to be completed on the same day of uterine flush. In some horse breeds, like Polo Argentino, females are preferred to males due to their higher abilities for performance

77 in polo.

78 Therefore, the aim of this work was to study if 1) the sex of in vivo and in vitro produced equine
79 embryos can be determined by amplification of DNA from blastocoele fluid, 2) the blastocoele
80 fluid of preimplantation equine embryos contain nuclear DNA and 3) the DNA present in
81 blastocoele fluid is originated by apoptosis.

82

83 **2. Materials and Methods**

84 *Animal Care and Welfare*

85 The protocol (011/2013) for this study was approved by the Committee on the Ethics of Animal
86 Experiments of the Universidad Nacional de San Martín following the recommendations in the
87 Guide for the Care and Use of Animals of the National Institute of Health.

88

89 *2.1 Experiment 1: Gender determination by amplification of DNA from blastocoele fluid from in*
90 *vivo and in vitro produced equine embryos.*

91

92 *2.1.1 In vivo produced equine embryos*

93 Ovarian follicular development in estrous crossbreed donor mares was monitored daily using
94 ultrasound scanning. When the dominant follicle(s) reached ≥35 mm in diameter, the mares
95 were artificially inseminated once with fresh semen from a fertile stallion and were administered
96 1 mg of a GnRH analog (BioRelease deslorelin acetate, BET Pharm, Lex, KY, USA) i.m. for
97 ovulation induction. Fixed twice daily (7AM / 7 PM) ultrasound examinations of the ovaries were
98 continued to diagnose the occurrence of ovulation (Day 0).

99 Exactly on Day 8 after ovulation detection on each donor mare, they were placed in stocks and
100 embryos were non surgically collected as described previously by Losinno et al. [7] using sterile
101 Ringer Lactate as flushing media and in line embryo filter. The residual medium in the filter

was transferred to a sterile plastic Petri dish and the embryo was searched using a stereomicroscope under a laminar flow. Once the embryo was located, it was morphologically graded using the scale proposed by McKinnon and Squires [8]. Only morphologically normal (Grade 1) and blastocyst stage embryos were included in the study.

2.1.2 Equine embryos produced in vitro by Intracytoplasmic Sperm Injection (ICSI)

Ovaries were obtained from a pool of mares with unknown reproductive history from an equine slaughterhouse located 50 km from our laboratory. Oocytes were obtained by aspiration of follicles with a 19 G needle connected to an aspiration pump and matured in vitro for 18-22 h in 50-µL microdroplets under mineral oil of TCM199 supplemented with 1 mM glutamine, 0.19 mM sodium pyruvate, 5 µg/mL FSH (Bioniche), 100 ng/mL EGF, 100 ng/mL IGF-I and 10 % Fetal Bovine Serum (FBS) (Gibco, South America). After in vitro maturation, expanded cumulus-oocyte complexes were incubated in a 0.1 % hyaluronidase solution for 10 min and their cumulus cells removed by vigorous pipetting through a fine-bore glass pipette.

All oocytes with a visible polar body and an intact cytoplasm were selected for injection and ICSI was performed as described previously by Palermo et al. [9]. Briefly, sperm cells were immobilized by disruption of the plasma membrane with the injection pipette. Each oocyte was injected with one stallion sperm while held in place by the holding pipette. All injected oocytes were cultured in vitro in SOFm with 19 mM D-glucose with 10 % FBS at 38º C, in 7 % O_2 and 5 % CO_2 for 48 h [10]. At this time, oocytes were observed under light microscopy and all cleaved embryos were cultured for at least 5.5 more days.

2.1.3 Blastocoele fluid collection

In vivo or in vitro produced blastocyst stage embryos were placed in 50-µL microdroplets of Dulbecco modified Phosphate Buffered Saline without calcium and magnesium (Sigma)

supplemented with 10 % FBS and 50 µg/mL of gentamicin (working medium) under mineral oil, on an inverted microscope equipped with a Nikon-Narishige micromanipulation system. Embryos were held in place by suction of a holding pipette and the inner cell mass was placed 90° clockwise away from the holding pipette. Then, the embryo capsule of in vivo produced embryos or the zona pellucida of in vitro produced embryos was punctured with a beveled micropipette (9 µm ID, ORIGIO, Humagen Pipets, USA). All the blastocoele fluid was aspirated and, using the same micropipette, discharged on a 1-µL microdroplet of working medium. The microdroplet containing the blastocoele fluid was examined under the microscope for the presence of embryonic cells that could have been accidentally aspirated during the procedure. Only samples that did not contain any cells were included in the study. The blastocoele fluid aspiration procedure (Figure 1) was performed by manual suction, connecting the biopsy micropipette to a disposable syringe. The complete volume of the microdroplet containing the blastocoele fluid was transferred to a 0.2-mL DNAse-free tube containing 4 µL of DNAse-free water. The operators performing the blastocoele fluid collection wore lab coats, non-powdered gloves, masks and caps. The micropipettes used for blastocoele fluid collection were replaced after each procedure, so that each set of pipettes was used for only one embryo. The tips used to handle blastocoele fluid samples were sterile, disposable, with filter and were used individually for each sample.

2.1.4 Amplification by PCR of the DNA present in the blastocoele fluid

A duplex PCR was performed to amplify the Y-encoded testis-specific protein (*TSPY*) and amelogenin (*AMEL*) genes. The oligodeoxynucleotide primers used to amplify these two sequences and the size of the expected amplification products are shown in Table 1. The total reaction volume was 12.5 mL and contained 6.25 mL MasterMix (Promega, Mi, USA), 0.2 µM *TSPY* primers, 0.1 µM *AMEL* primers, and 3.25 µL of the sample. The thermal cycling

parameters were 95 °C for 1 min, 35 cycles of 94 °C for 15 s, 58.4 °C for 30 s, 72 °C for 30 s; and 72 °C for 5 min. The PCR was performed twice, using the same primers and cycling protocol. In order to monitor a possible DNA contamination, one tube was included as a control in the first PCR and contained all the necessary components for the PCR, except 3.25 µL of DNAse-free water were placed in the tube instead of the DNA sample. In the second PCR, 3.25 µL of the amplification product from the first PCR was used as DNA template. All the samples and reagents for PCR and the amplification products were handled using sterile disposable tips with filter. The amplification products were electrophoresed on a 2% agarose gel stained with ethidium bromide and visualized under UV light.

Table 1. Primers used for sex determination of equine embryos by amplification of DNA in blastocoele fluid.

Primer	Sequence	Fragment size (bp)	References
AMEL-F	5'-CCAACCCAACACCACCAGCCAAACCTCCCT-3'	184 (female) 160/200 (male)	Hasegawa et al [11]
AMEL-R	5'-AGCATAGGGGGCAAGGGCTGCAAGGGGAAT-3'		
TSPY-F	5'-GAA GTC AGG CAC ACC AGT GA-3'	280	Paria et al [12]
TSPY-R	5'-TAA GGC TGC AGT TGT CAT GC-3'		

2.1.5 Survival of in vivo produced embryos after blastocoele fluid aspiration and in vitro culture

Immediately after blastocoele fluid aspiration, seven in vivo produced embryos were placed individually in 50-µL microdroplets of SOFm with 19 mM D-glucose with 10 % FBS under mineral oil at 38º C, in 7 % O_2 and 5 % CO_2 and observed at 24, 48 and 72 h. The diameter of embryos was registered immediately before blastocoele fluid aspiration and every 24 h during in vitro culture.

172

173 *2.1.6 Amplification by PCR of the DNA from sampled embryos*

174 After blastocoele fluid aspiration or blastocoele fluid aspiration and in vitro culture for 72 h, all
175 embryos were sliced into small fragments using a micro-blade and each fragment was placed in
176 a 0.2-mL DNAse-free tube with 4 µL of DNAse-free water and maintained at -20 °C until
177 analyzed by PCR. The primers and cycling protocol were the same as with the blastocoele fluid
178 samples. Results of the amplification of the DNA from sampled embryos were compared with
179 the results of the amplification of the DNA from the blastocoele fluid samples from the same
180 embryos.

181

182 *2.2 Experiment 2: Genetic sequence analysis of the amplified DNA from blastocoele fluid of in*
183 *vivo produced embryos*

184 The band on the agarose gel corresponding to the amplification product of the expected
185 molecular weight of the specific PCR using TSPY primers from two different samples obtained
186 from in vivo produced embryos was extracted using the QIAEX DNA extraction kit (Qiagen, CA,
187 USA), according to the manufacturer's recommendation. The purified fragments were cloned
188 into pGEMT-easy (Promega) and subsequently propagated in *Escherichia coli* TOP TEN
189 (Invitrogen, CA, USA). Bacteria were grown in Luria-Bertani medium (10 g Bacto tryptone, 5 g
190 yeast extract and 10 g NaCl in 1L water) overnight at 37 °C with stirring. At 18 h of culture, a
191 midiprep was performed with 150 mL of medium using the Qiagen midi kit (Tip 100, Qiagen),
192 following the manufacturer's recommendations. The plasmids obtained were quantified by
193 spectrophotometry and sequenced with SP6 and T7 primers. Sequencing was performed by an
194 ABI PRISM 3111 sequencer. Sequences were deposited at the National Center for
195 Biotechnology Information (NCBI) and compared using the Basic Local Alignment Tool (BLAST)
196 with existing sequences at GenBank. Only sequences with more than 99 % of identity were

accepted.

2.3 Experiment 3: DNA ladder assay of blastocoele fluid from in vivo produced embryos

Blastocoele fluid from three different Day-8 in vivo produced equine embryos was obtained as described in Experiment 1. DNA was purified from blastocoele fluid samples through minispin kit columns (Qiagen), following the manufacturer's protocol. Electrophoresis was performed in 10 % polyacrylamide gel in 1X TBE buffer (89 mM Tris, 89 mM Boric Acid, 2 mM EDTA) for 2 h at 100 Volts.

After electrophoresis, the tray was disassembled and the gel was washed with purified water for 10 s. Then, the gel was incubated for 10 min in fixing solution (acetic acid in water 7.5 % v/v). Once completed, the gel was washed with deionized water for 2 min. Additionally, a pre-treatment was performed with a solution of formaldehyde (15 % in water v/v) for 10 min, then incubated for 20 min with a silver solution (0.1 g $AgNO_3$ in water) and washed twice with deionized water for 3 s. The gel was revealed by incubation in developing solution (3 g Na_2CO_3, 1 mM sodium thiosulfate in 100 mL of water). The reaction was stopped with stop developer solution (7.5 % acetic acid in water).

3. Results

3.1 Experiment 1

Thirteen in vivo and five in vitro produced equine embryos were used for this experiment. The bands observed after PCR and electrophoresis of the amplified female and male samples are shown in Figure 2. The genetic sex was determined in 11 out of the 13 (84.6 %) and 4 out of the 5 (80 %) blastocoele fluid samples obtained from in vivo and in vitro produced embryos

respectively. Eight out of the 11 blastocoele fluid samples from the in vivo produced embryos that amplified were diagnosed as males and three were diagnosed as females, whereas two out of the four amplified samples from the in vitro produced embryos were diagnosed as males and two as females. The results obtained by PCR of blastocoele fluid were compared with those obtained after PCR of fragments of embryos from which the blastocoele fluid was collected. The sex diagnosed from blastocoele fluid samples was the same as that diagnosed from the corresponding pieces of embryos in all 15 cases (11 in vivo and 4 in vitro produced embryos respectively). The two blastocoele fluid samples from in vivo produced embryos and the only sample from an in vitro produced embryo that failed to amplify after PCR were obtained from female embryos.

Seven of the thirteen in vivo produced embryos were cultured in vitro and observed every 24 h for post- blastocoele fluid aspiration survival. Six out of seven embryos reexpanded at 24 h of in vitro culture and increased their diameter at 48 and 72 h. The average percentage increase in diameter at the end of the culture period was 73.7%, from a mean of 633 µm to a mean of 1100 µm. Four of the six reexpanded embryos hatched in vitro during the culture period. Figure 3 shows one of the in vivo produced embryos hatching, after 48 h of in vitro culture.

3.2 Experiment 2

The sequence cloned into pGEMT-easy and plasmid DNA was isolated and purified by spin Qiaprep miniprep kit (Qiagen, CA, USA), according to the manufacturer's protocol. Then, the plasmid DNA was compared using BLAST. The fragment obtained after PCR of DNA in the blastocoele fluid was 100 % identical to a sequence in the Y chromosome of *Equus caballus*, sequence ID: AC215855.2, demonstrating that the amplified sequence was genomic DNA.

3.3 Experiment 3

This experiment was repeated three times, using blastocoele fluid from three different in vivo produced equine embryos. Separation of DNA from blastocoele fluid by polyacrylamide gel electrophoresis and silver staining showed a ladder pattern in all three samples, characteristic of DNA from cells undergoing apoptosis (Figure 4).

4. Discussion

Preimplantation Genetic Diagnosis, which allows the detection of genetic traits in early embryos, is a well-established procedure that involves the collection of one or more cells. Recently, Palini et al. [2] demonstrated that PGD could also be performed using blastocoele fluid of in vitro produced human preimplantation embryos, obtained prior to vitrification.

In the present work, we confirmed the findings of Palini et al. [2] by detecting the sex of in vitro produced equine embryos and showed that this strategy can be applied to in vivo produced equine embryos as well, using conventional PCR.

Although we were able to diagnose the sex of 11 out of the 13 in vivo produced embryos and of 4 out of the 5 in vitro produced embryos, this technique failed to produce a result in three of the samples. The diagnosis rate of this technique was high, but further studies with a larger number of samples will demonstrate whether this efficiency can be improved. Using multi-copy genes for PCR improves the detection rate in samples with low amounts of DNA, such as those of blastocoele fluid. The use of blastocoele fluid for PCR is probably limited to multi-copy genes and not suitable for the detection of other genes that are in single copy. This was evidenced in Experiment 1, where all three samples that failed to produce an amplification product were from female embryos, demonstrating that *AMEL* - being a single-copy gene - was not efficiently detected. All male samples were diagnosed successfully, using specific primers that targeted the *TSPY* gene, which is involved in testicular development and is present in multiple copies in

the equine genome of male samples [12].

Because of the small amount of DNA present in blastocoele fluid, a double PCR and primers targeting multicopy genes need to be used to achieve amplification. This methodology is highly sensitive to DNA contamination and therefore, handling of blastocoele fluid samples and amplification products needs to be performed with extreme caution. Operators performing the PCR have to wear proper protection, samples and amplification products need to be handled using tips with barriers and negative controls have to be included on each step of the protocol. In Experiment 2, by using specific primers targeting for *TSPY* and genetic sequencing, we were able to confirm that the amplified sequence was from *Equus caballus* DNA located in the Y chromosome. This result confirms that the DNA in blastocoele fluid used as a template for PCR was nuclear DNA.

In Experiment 3, we demonstrated that the DNA present in the blastocoele fluid from in vivo produced embryos is fragmented and shows a characteristic ladder pattern present in cells undergoing apoptosis. To our knowledge, this is the first report presenting evidence that the DNA in blastocoele fluid is originated from apoptosis. Tremoleda et al. [13] showed that equine embryos produced in vitro by ICSI present a much higher number of apoptotic cells than in vivo produced embryos, with only 4 out of 10 in vivo produced embryos presenting apoptotic cells. In Experiment 1, we were able to amplify the apoptotic DNA present in blastocoele fluid by PCR, proving that in vivo produced embryos present enough apoptotic DNA as a template for PCR. Previous reports have shown that blastomere biopsy of murine embryos produce epigenetic aberrations in the brain tissue of the offspring [14] and that there is an abnormal development and function of neurons of mice generated after blastomere biopsy [15]. Blastocoele fluid collection could be less detrimental to the embryo because no embryonic cells are extracted during the procedure, is technically simpler than conventional PGD and is performed in less time. In the present study we observed that a high proportion of aspirated embryos survived and increased their diameter after in vitro culture for 72 h. Although the pregnancy rates of in vivo or

in vitro produced equine embryos transferred after blastocoele fluid extraction still needs to be studied, the in vitro survival of aspirated embryos demonstrates this approach does not impair their viability. Since it has already been shown that the pregnancy rates of biopsied or intact in vivo produced equine embryos are not significantly different [16, 17], similar results are expected using embryos from which only blastocoele fluid and no cells are obtained. In a previous report, we aspirated ten to thirty blastomeres from each biopsied embryo [17] and embryos collapsed completely during the biopsy procedure, similar to that observed after blastocoele fluid collection.

5. Conclusions

The results presented in this study demonstrate that the sex of in vivo and in vitro produced equine embryos can be diagnosed using blastocoele fluid as the DNA source for conventional PCR. Our results also confirm that the DNA amplified by PCR is nuclear DNA and that fragmented DNA, probably originated from apoptosis of embryonic cells, is present in the blastocoele fluid of in vivo produced embryos. The large size and consequently, the large volume of blastocoele fluid present in equine embryos, make the horse a suitable animal model to study the presence of apoptotic DNA during early development.

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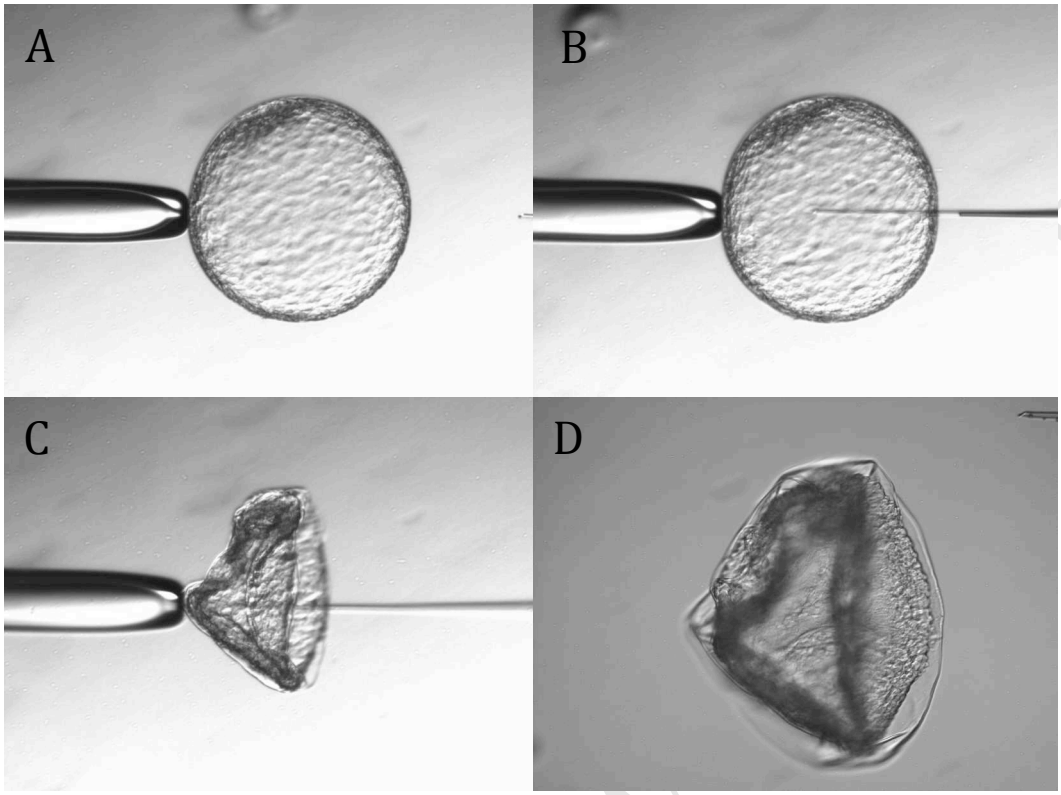
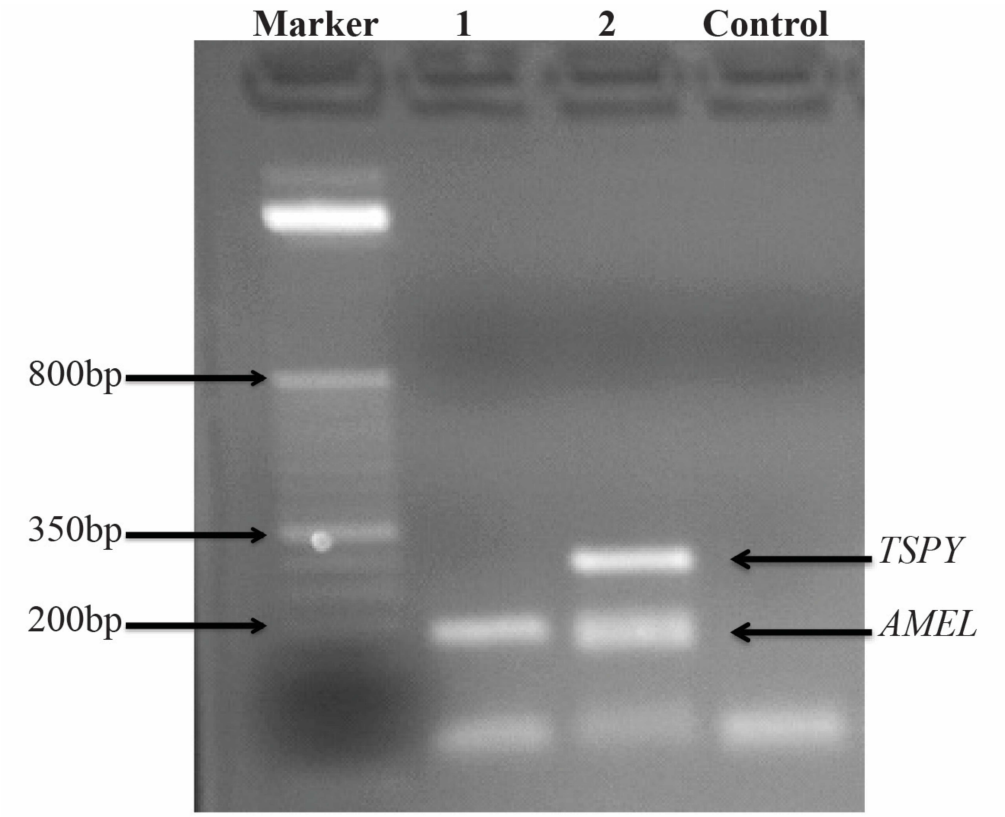
Figure 1. Blastocoele fluid collection from an in vivo produced equine embryo 500 µm in diameter. A: In vivo produced equine embryo before blastocoele fluid aspiration; B: The embryo is punctured with the aspiration micropipette; C: The blastocoele fluid is aspirated; D: The same embryo as in A after blastocoele fluid collection.

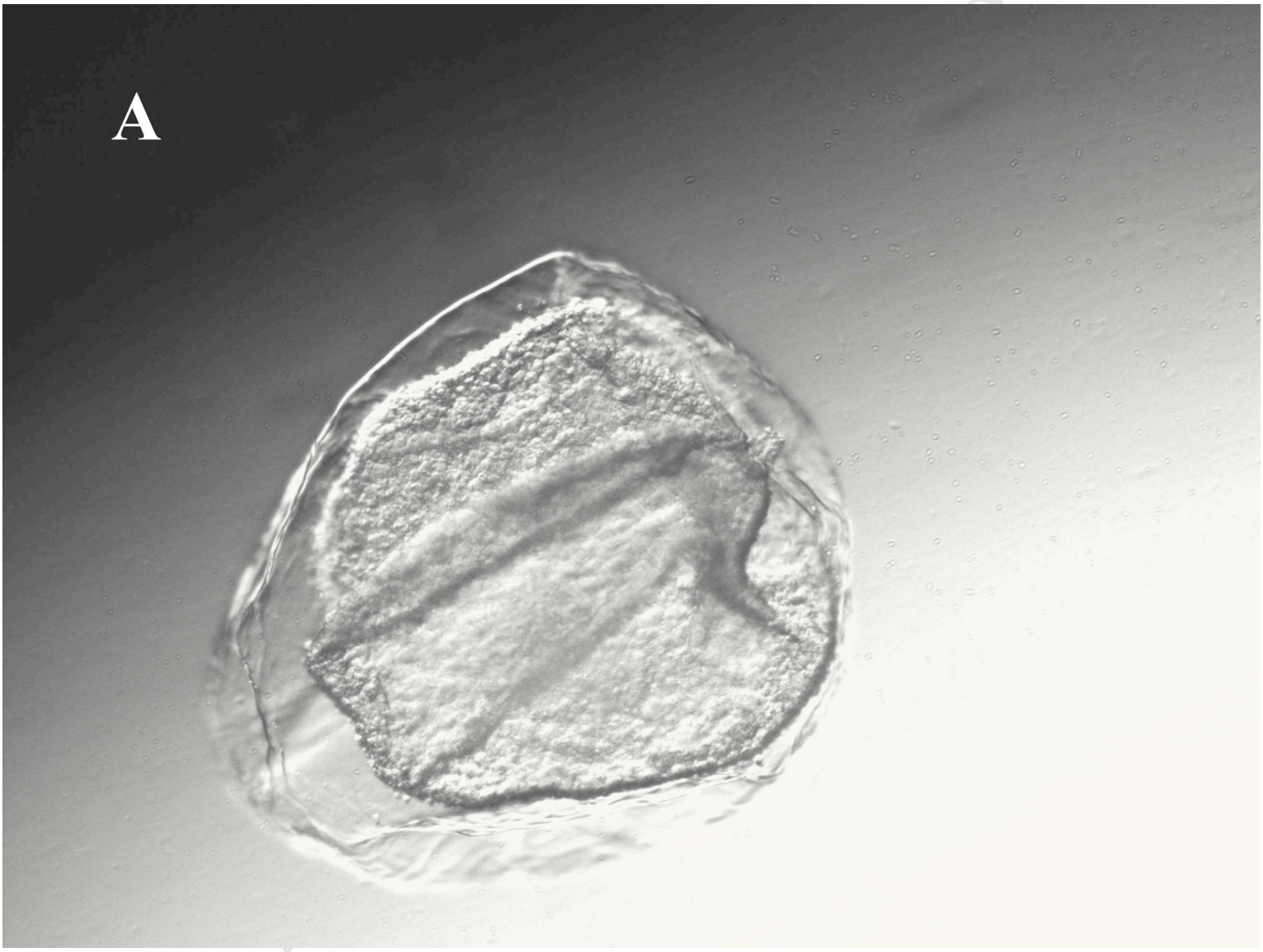
Figure 2. Electrophoresis in agarose gel of *TSPY* and *AMEL* PCR products. M: 50-bp marker; 1: Female sample, one *AMEL* band at 184 bp; 2: Male sample, one *TSPY* band at 280 bp and two *AMEL* bands at 160 bp and 200 bp; 3: No DNA control.

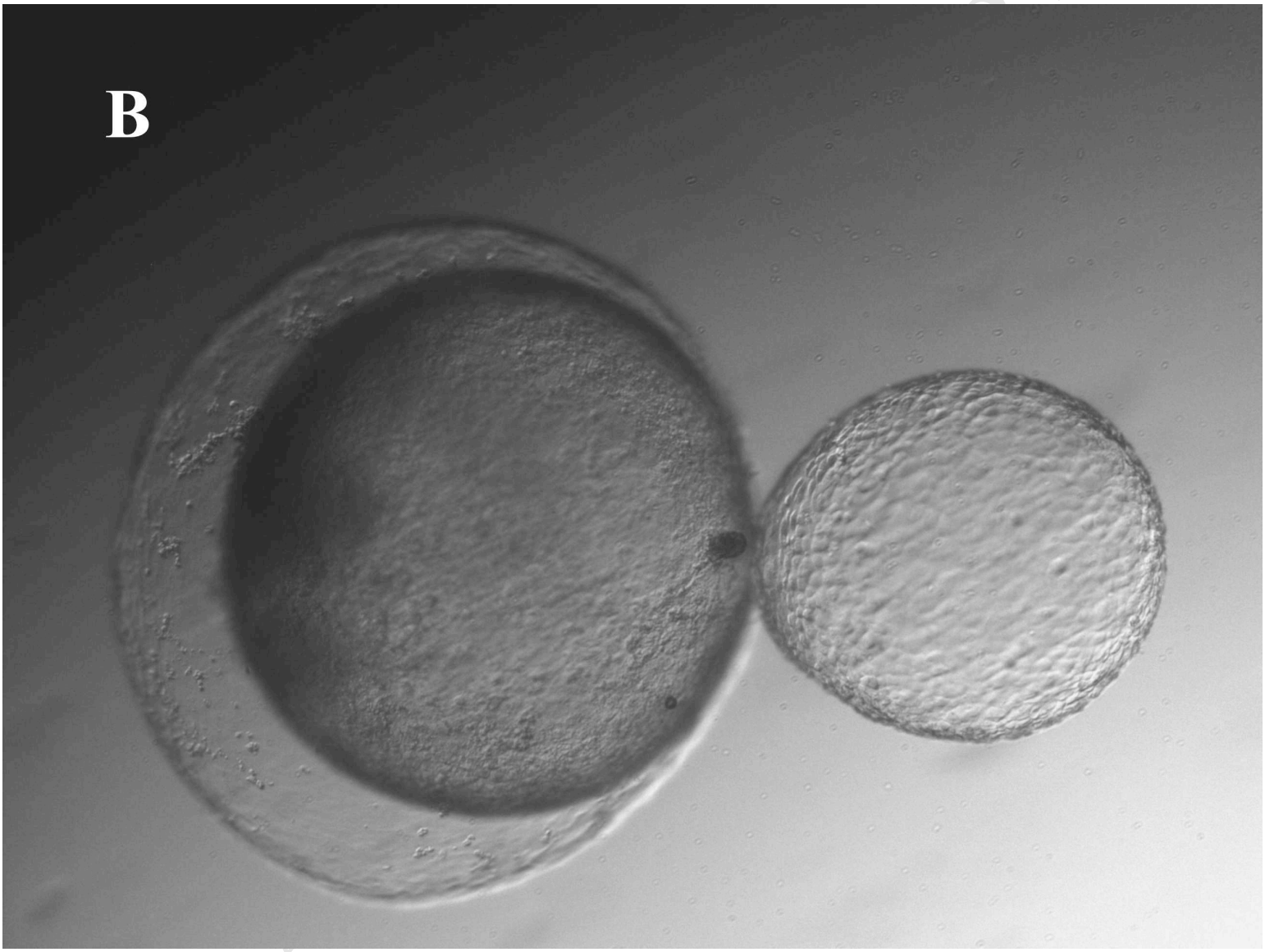
Figure 3. In vivo produced equine embryo. A: After blastocoele fluid aspiration; B: After 48 h of in vitro culture.

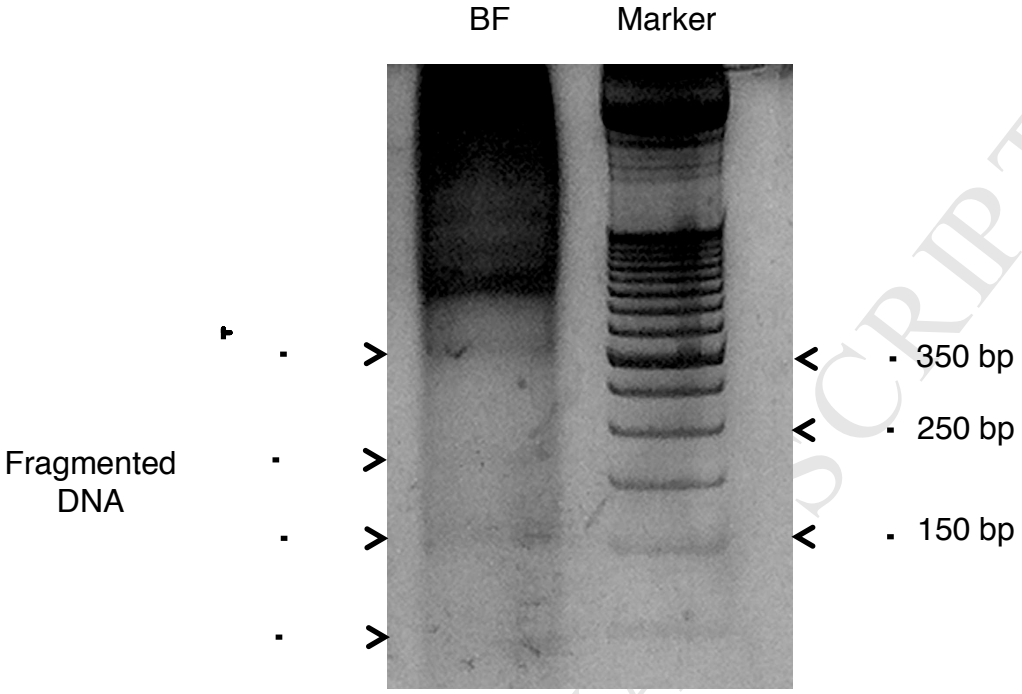
Figure 4. Polyacrylamide gel electrophoresis and silver staining of blastocoele fluid from an in vivo produced equine embryo. BF: Blastocoele fluid; M: 50-bp marker.






A


B



Highlights

- Blastocoele fluid of equine preimplantation embryos contains genomic DNA.
- The DNA present in blastocoele fluid is probably originated by apoptosis of blastomeres.
- Blastocoele fluid contains enough DNA to diagnose preimplantation embryos by PGD.
- Aspiration of the blastocoele fluid does not impair embryo viability.